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A new *Graphilbum* species from western hemlock (*Tsuga heterophylla)* in Canada

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Abstract — A new species of *Graphilbum*, *G. tsugae* isolated from *Tsuga heterophylla*, is described from British Columbia, Canada. Although rDNA ITS data analysis demonstrates its close relationship to *Graphilbum rectangulisporium*, morphologically the unique apical projections arising from the perithecial necks of *G. tsugae* easily differentiate it from the former and all other described *Graphilbum* and *Ophiostoma* species. We also correct the epithet orthography and the parenthetical basionym author citation of the name *G. rectangulisporium*.

Key words — ophiostomatoid fungi, bark beetles, morphology, taxonomy, ITS rDNA region.

Introduction

During a reassessment of isolates of ophiostomatoid fungi in our culture collection whose taxonomic placements had never been resolved, we examined three strains from a single location in southern British Columbia isolated from two felled logs of *Tsuga heterophylla*. These logs had shown clear evidence of having been attacked by bark beetles — many bore holes were evident and peeling back the bark around the holes revealed well-developed galleries — but neither beetle adults nor larvae were present and the surrounding sapwood was only slightly stained. However, hand lens examination did show both remnant hyphae and possible reproductive structures scattered throughout gallery interiors.

Slabs of bark-covered wood about 12 inches long, 6–8 inches wide, and 1–3 inches thick were harvested from such areas using a hatchet and a chisel. The bark was then removed to expose the outer sapwood surfaces and the inner bark surfaces of these slabs. After the wood slabs and bark pieces were examined carefully to ensure no adult beetles or larvae were present in any of the exposed galleries, these materials were placed and sealed in collection bags for return to the laboratory.

When we began our detailed study of this species, the *Ophiostomatales* accommodated three genera: *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr.; *Grosmannia* Goid.; and *Ophiostoma* Syd. & P. Syd., among which species economically important tree pathogens and blue-stain fungi are found (Olchowecki & Reid 1974, Wingfield et al. 1993, Hausner et al. 2005).

Based on partial LSU rDNA and β-tubulin sequences, Zipfel et al. (2006) provided some support for the circumscription of the above three genera. But Zipfel et al. (2006, p. 95) and other authors (Hafez et al. 2012, 2013) also noted that the use of molecular criteria might require the erection of new genera to accommodate either previously misplaced or subsequently newly discovered species of *Ophiostoma* sensu lato.

Recently de Beer & Wingfield (2013), using partial LSU and SSU rDNA sequences, have begun redefining the *Ophiostomatales*. They include only one family, the *Ophiostomataceae*, in which they recognize six distinct lineages represented by the genera *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. emend. Zipfel et al. (2006), *Fragosphaeria* Shear, *Leptographium* Lagerb. & Melin sensu lato (includes *Grosmannia*), *Ophiostoma* sensu lato (includes *Pesotum* and *Sporothrix)*, *Raffaelea* Arx & Hennebert emend. T.C. Harr. in Harrington et al. (2008), and *Graphilbum* H.P. Upadhyay & W.B. Kendr. emend. Z.W. de Beer et al. (2013a). They also clearly implied the possible need for further generic inclusions. Relevant comments are also found in related accompanying papers (de Beer et al. 2013a & b).

The genera of the *Ophiostomataceae* include economically important tree pathogens and blue-stain fungi (e.g., Lagerberg et al. 1927, Olchowecki & Reid 1974, Wingfield et al. 1993, Hausner et al. 2005) whose members lack forcible ascospore discharge, have deliquescent asci, develop sticky ascospore droplets at the apex of their perithecial necks, and typically produce slimy/sticky conidia on various simple or complex conidiophore structures. Species assigned to *Ceratocystiopsis* resemble those of *Ophiostoma* except in *Ceratocystiopsis* the small dark perithecia only develop short perithecial necks and produce falcate, sheathed ascospores (Upadhyay 1981, Zipfel et al. 2006, Plattner et al. 2009). These species also have a lower tolerance to cycloheximide than do *Ophiostoma* species (Harrington 1981, Hausner et al. 1993). Species formerly assigned to *Grosmannia* (above) were distinguished by the fact they have *Leptographium* states.

As we could not definitively establish the identity of our three isolates using only morphological criteria, we compared nuclear small subunit rDNA (rSSU) and rDNA internal transcribed spacer (ITS) region (ITS1, 5.8S gene, and ITS2) sequences from our isolates with those of other *Ophiostoma* species. We also assessed the growth rates and cultural characteristics of our isolates at different temperatures and compared our results with data published for other species. We did not assess mating type features, as we had earlier when describing another ophiostomatoid species (Reid et al. 2010), as mating type studies had not been conducted for any of the species with which we compared our current isolates.

Based on the revisions discussed above, we believe our results show that our isolates represent a new taxon within the genus *Graphilbum* that we describe here as *Graphilbum tsugae*.

Materials & methods

Isolation of strains studied, culturing procedures, and growth-temperature experiment

Laboratory isolations were made from field collected wood and bark samples and grown on agar plates in a 20°C incubator; these were examined periodically for evidence of fungi of interest.

We obtained three isolates that, based solely on morphological criteria, appeared to represent an undescribed *Ophiostoma* species. Strains examined and/or sequenced are detailed in Table 1. Living cultures and dried specimens of our new species have been deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Canada; the additional DNA sequences retrieved from GenBank employed in the analyses are also listed in Table 1.

Based on the intent of the experiment, strains were grown and characterized employing either malt extract agar supplemented solely with yeast extract (Reid et al. 2010) or such agar that also contained wood chips (Reid & Hausner 2010); fertile perithecia were regularly produced on the latter plates. These were then dried, and one was selected to be the type specimen. The parent culture was isolate WIN(M) 1391, obtained from a perithecial spore drop produced at the apex of a perithecium found in the gallery of an unidentified bark beetle. Preparations for microscopy were mounted in 85% lactic acid and at least 50 measurements were made for each significant morphological feature. Colour designations are based on Rayner (1970).

In a previous study (Reid et al. 2010) where we had found a group of very similar isolates growing on pieces of substrate in very close proximity to each other — as was the case here — we had processed three of them in a temperature growth-rate response experiment, and they produced very similar growth-temperature files. They and the other isolates in the group were described as *Ceratocystis norvegica* J. Reid & Hausner [sic; = Georg Hausner].

Because of that experience, the precise identity of the gene regions sequenced in our current three isolates, and the lack of any significant morphological variation noted between them, we used only the designated holotype isolate, UAMH 11701 [WIN(M) 1391] for determining the temperature growth-rate response of *G. tsugae*.

We employed the protocols of van Wyk et al. (2006), as modified by Reid et al. (2010). However, because of the cultures' relatively slow growth, the first measurements were not made until the second day after plate inoculation. We used eight replicate plates at each test temperature. Although initially, the total temperature-growth test

Table 1. The rDNA ITS region sequences evaluated

Taxon	Strains/Isolates/source[1]	GenBank
Ceratocystiopsis minuta	[2]CBS 145.59	DQ128173
C. cf. *minuta*	SL-K 70	DQ128175
Grosmannia penicillata	RJ-T 0125	AM943882
Ophiostoma abietinum	CMW 397	DQ396788
O. dentifundus	CMW 13016 [a]	AY495434
	CMW 13017 [a]	AY495435
O. ips	CMW 22843	DQ539549
O. grandicarpum	Strain D	AJ293884
O. microcarpum	YCC 459	AB506676
	YCC 612	GU134170
O. piceae	Ci-37	EF506934
O. pulvinisporum	CMW 9026	AY546715
O. rectangulisporium	TFM:FPH 7756	AB242825
	YCC 617	GU134171
O. cf. *rectangulisporium*	CMW 26261 [b]	EU785451
	CMW 26259[b]	EU785450
	CMW 26258 [b]	EU785449
	C2477	GU129987
	C2300	GU393357
	253GRJ [c]	JX444645
	1313RJP [c]	JX444594
	1039RJP [c]	JX444590
	1037RJP [c]	JX444589
	1284RJP [c]	JX444591
	1308RJP [c]	JX444593
	1306RJP [c]	JX444592
	246bGRJ	JX444644
	CMW 22830 [d]	DQ539536
	CMW 22829 [d]	DQ539535
	CMW 22832 [d]	DQ539538
	CMW 22831 [d]	DQ539537
	1132RJ [d] (=voucher 1138RJ)	JQ289021
O. rostrocoronatum	CBS 434.77	AY194509
Graphilbum tsugae	WIN(M) 1391 [e] (=UAMH 11701)	KJ661745
	WIN(M) 1384 [e] (=UAMH 11700)	—
	WIN(M) 1387 [e] (=UAMH 11699)	—
Ophiostoma sp.1	WIN(M) 1602	HM363177
	WIN(M) 1603	HM363164
Ophiostoma sp. 2	RJ-0771 [f]	AM943895
	RJ-0704 [f]	AM943894
Ophiostoma sp. 3	PR-2006c (=CMW 27315)	DQ674367

Pesotum fragrans	CMW 22853	DQ539561
	C01-014a ^{g}	AY194513
	NLC 348 ^{g}	AY194518
	CBS 279.54 ^{g} (=C1224)	AF198248
	WIN(M) 1388 ^{g}	—
	C990	DQ062976
P cupulatum	C1194	AF198230
Sporothrix inflata	CMW 12529	AY495428

[1] Strain numbers followed by identical superscript letters (^{a} to ^{g}) have identical ITS regions and only one representative was used in the ITS phylogenetic analysis.

[2] CBS = CBS-KNAW Fungal Biodiversity Center, Utrecht, Netherlands; WIN(M) = culture collection of J. Reid, Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada; UAMH = University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Edmonton, Alberta, Canada; CMW, Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; STE-U, Stellenbosch University, South Africa.

period was to have lasted seven days, we incubated all plates further at 20°C to record morphological development.

DNA extraction, amplification protocols and DNA sequencing

DNA extraction, purification, and agarose electrophoresis protocols followed Hausner et al. (1992). Genomic DNA served as the amplification template using the Invitrogen-Life Technologies PCR System (Buffer and Taq polymerase, Invitrogen, Fredrick, MD). Primers SSUZ and LSU4 (Hausner et al. 2005) were used to amplify the ITS regions. The PCR primer sequences, amplification conditions, sizes of the expected PCR products, and preparation of sequencing templates for fragments have been described previously (Hausner & Wang 2005, Hausner et al. 2005). DNA sequencing templates were prepared with the aid of the Promega Wizard SV Gel and PCR clean-up system (Promega, Madison, WI). Purified double-stranded PCR products were sequenced in both directions using the cycle-sequencing protocols performed according to the manufacturer's recommendations (Perkin Elmer Applied Biosystems, Foster City, CA), and automated Fluorescent DNA sequence analysis was performed using an ABI Prism 310 Genetic Analyzer system (PEAB at the University of Calgary, DNA sequencing facility, Calgary, AB).

Analyses of DNA sequence data

To add to sequences generated herein, we used the UAMH 11701 ITS sequence in a BLAST search to recover additional sequences from NCBI databases for comparison with other relevant taxa. Fifty ITS rDNA sequences were aligned with CLUSTAL-X (Thomson et al. 1997) and, when appropriate, modified with the alignment editor program GeneDoc v2.5.010 (Nicholas et al. 1997, http://www.psc.edu/biomed/genedoc). Phylogenetic trees were generated using the Molecular Evolutionary Genetic Analysis program package (MEGA 5.1; Tamura et al. 2011). Trees were generated with the Maximum parsimony (MP), Neighbor joining (NJ), and Maximum likelihood (ML) methods. MEGA 5.1 was also used for determining the best-fit DNA models and the best-fit substitution model for ML analysis. In order to evaluate node support within the tree topologies, the bootstrap option (1000 replicates) was selected for all tree-building programs.

Finally, MrBayes (version 3.1; Ronquist & Huelsenbeck 2003) was used for Bayesian analysis. The NEXUS file format necessary for the alignment (input) file was generated with the file converting option available within DAMBE (Xia 2001). The DNA substitution model setting for Bayesian analysis was chosen based on evaluating the ITS region alignment with the Modeltest 3.7 program (Posada & Crandall 1998). The Bayesian inference of phylogenies was initiated from a random starting tree, and four chains were run simultaneously for 3 million generations; trees were sampled every 100 generations. The first 25% of trees generated were discarded (burn-in); the remaining trees were used to construct a 50 % majority rule consensus tree and compute the posterior probability values.

Phylogenetic trees were drawn with the TreeView program (Page 1996) and figure annotations added using Corel Draw (Corel Corporation and Corel Corporation Limited).

Results

Cultural and morphological characteristics

The temperature-related growth response of isolate UAMH 11701 (Fig. 1) depicts a relatively broad growth temperature optimum, i.e. 20–25°C, dropping off sharply towards both 15 and 30°C. The mycelium amount produced differed between test temperatures but was always appressed, with only limited sparse, central aerial mycelium. During the first 7 days, the mycelium remained hyaline (translucent) and without coloration or reproductive structure development.

All eight plate sets from the various test temperatures were then placed in a single 20°C incubator.

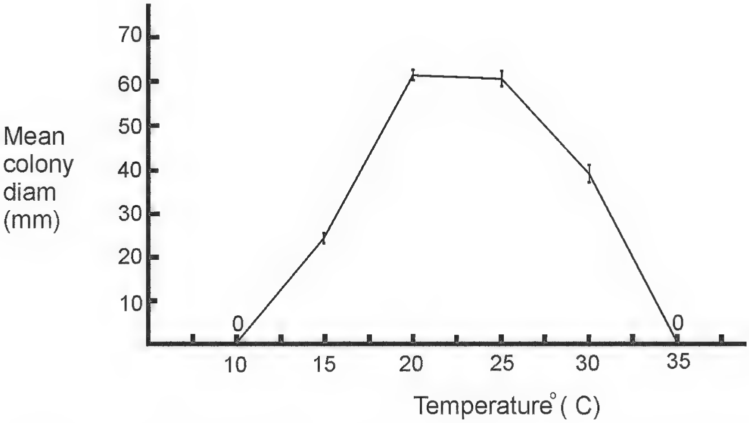


Fig. 1: Temperature-growth curve for *Graphilbum tsugae* holotype strain UAMH 11701 incubated at various temperatures for 7 days.

By day10, mycelium in all original 20°C and 25°C cultures had reached Petri dish margins. It was still mostly hyaline and appressed, but with a ring of slightly more floccose white-colored mycelium around the paler center (Fig. 2A); these cultures were virtually identical in growth rates and appearance.

The 15°C and 30°C cultures had also grown further; their diameters were still quite small, but the 10°C and 35°C cultures showed only numerous short hyphae extending from their inoculation discs; apparently the fungus could survive a short exposure to the relatively high 35°C temperature.

All plate sets were then returned to the 20°C incubator until day 17.

In all original 20°C cultures, the mycelium had completely covered the dish surfaces. It was still quite peripherally appressed, more floccose centrally, and while still primarily white, there were definite scattered amber tints and a central darkening (Fig. 2B). Very occasionally scattered immature differentiating perithecia were observed, but only a few showed what might have been developing neck initials.

On day 17 in the original 25°C cultures, the mycelium had also reached dish margins. Generally it was still white, but now there were scattered slight amber tints. Less aerial mycelium was present peripherally than centrally, but that difference was now less pronounced. Overall there was less aerial mycelium than in the 20°C plates, but the central dark (Fuscous Black) pigmentation (Fig. 2C) was more pronounced and perithecial development more advanced. In six dishes the perithecia were quite numerous but fewer in the remaining two, suggesting that 25°C may be slightly better for perithecial production.

On day 17 in the original 10°C cultures, all isolates were growing, but no mycelium had reached a dish margin. Colony diameters averaged 70 mm, the mycelium was white and peripherally appressed to slightly floccose centrally, but no perithecial initials were present. In the original 15°C cultures, mycelium, which reached the dish margins randomly, was white, appressed peripherally, and slightly floccose centrally.

On day 17 in the original 30°C cultures, mycelium had reached the dish margins uniformly, but again the amounts of central and peripheral mycelium differed markedly. The peripheral mycelium was white and appressed while the central mycelium was more floccose and showing numerous presumptive perithecium initials. And while all original 35°C cultures were growing, no mycelium had reached a dish margin. Colony diameters averaged 53 mm, and in every case the mycelium was white, appressed peripherally and more floccose centrally, and there was no evidence of perithecia.

After inspection, we retained only the original 20°C and 25°C cultures, which were thereafter both maintained at 20°C.

By day 24, these retained cultures were similar in appearance. New mycelium was less dense, older mycelium had collapsed centrally, and scattered amber

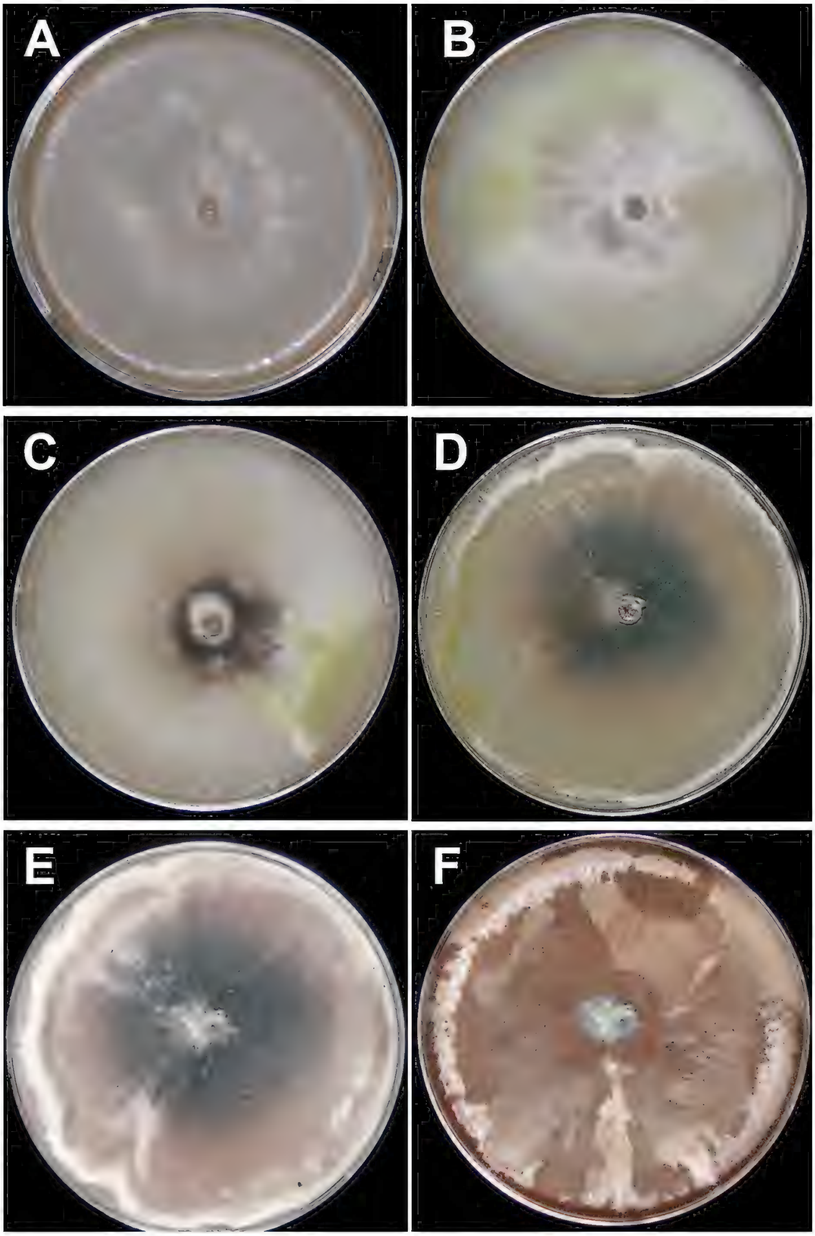


Fig. 2. *Graphilbum tsugae* (holotype, UAMH 11701 [WIN(M) 1391]). Morphology after growth at various temperatures. A. Original 20 °C plate at day 10. B. Original 20 °C plate at day 17. C. Original 25 °C plate at day 17. D. Original 25 °C plate at day 24. E. Original 25 °C plate after c. 7 weeks; F. Original 20 °C plate after c. 7 weeks.

colored patches, which were more common, often had turned pale yellowish-brown. More dark pigment was present centrally in the agar, and mounds of floccose white hyphae had formed at the colony margins. Although many perithecial initials were seen, no mature perithecia were found (Fig. 2D, an original 25°C culture).

Fig. 2E illustrates changes that occurred in an original 25°C culture over a further period of approximately 6–8 weeks. Note the increased amount of marginal mounded mycelium, increased area of central dark pigmentation, slight further overgrowth of superficial aerial mycelium, and finally, what appear to be abundant immature perithecia. Fig. 2F shows an even older original 20°C culture with extensive development of — now collapsed — marginal mounded mycelium but no apparent superficial mycelial overgrowth; there was also a restricted central dark pigmentation. Numerous perithecia were produced, but we only rarely saw any evidence of sporulation. Figs. 2E–F both show an extensive development of cinnamon-colored pigment throughout.

Because we rarely observed fully mature perithecia exuding spore droplets on agar dishes, we grew the strains on agar plus wood chips where mature perithecia were readily produced and their nature could be fully characterized (see Table 2 and Taxonomy section).

Table 2. Morphological comparisons between *G. rectangulisporium* and *G. tsugae*

Character	*Graphilbum rectangulisporium* [a]	*Graphilbum tsugae*
Perithecial base		
Shape	Globose	Obpyriform, ampulliform or globose [b];
Width (µm)	70-110	75-120(-167.5)
Height (µm)	70-110	75 -113(-140)
Perithecial necks		
Length (µm)	190-500 including ostiolar hyphae	(87-)138-200(-280) including ostiolar hyphae
Base width (µm)	20-35	27.5-50
Tip width (µm)	10-19	12.5-25
Ostiolar hyphae		
Length (µm)	13–43	(20-)25-40
Number	5-15	Various
Shape (µm)	Cylindrical with apical taper	Crooked to jagged in outline; at times irregularly cellular; tapering to a point.
Ascospores (µm)	2.0-3.5 × 1.0-1.7 with sheath; rectangular in side and face view	3-5 × 1 with sheath; oblong to rectangular; sheath flaring at corners
Anamorph	None found	Conidiophores simple to branched; not identifiable to genus; Conidia hyaline, 1-celled, oblong, 2-5 × 1-1.5 µm.
Colony color	White; remaining so on 2% MEA	White initially, coloring (pale yellowish brown) on aging on 2% MEA + 1% YE [c]

[a] Ohtaka et al. 2006; [b] Kirk et al. 2008; [c] Reid et al. 2010.

DNA sequence analysis

The rDNA SSU sequence (GenBank accession: HQ634824) for isolate UAMH 11701 — then as WIN(M) 1391 — was previously included in a study examining rDNA SSU intron distributions among ophiostomatoid fungi and related taxa (Hafez et al. 2012; p. 102). It showed that although UAMH 11701 was part of the *Ophiostomataceae* clade, it did not group with representatives of *Grosmannia*, *Ceratocystiopsis* or *Ophiostoma* sensu stricto. Instead, within the phylogenetic tree it assumed the most basal position among the sampled *Ophiostomataceae* taxa.

Our rDNA sequence analysis of the internal transcribed spacer (ITS) region (ITS1, 5.8S, and ITS2; KJ661745) yielded even more surprising results. Using this sequence set from isolate UAMH 11701 — again as WIN(M) 1391— in a BLASTN analysis, revealed that its sequences are similar to those from this

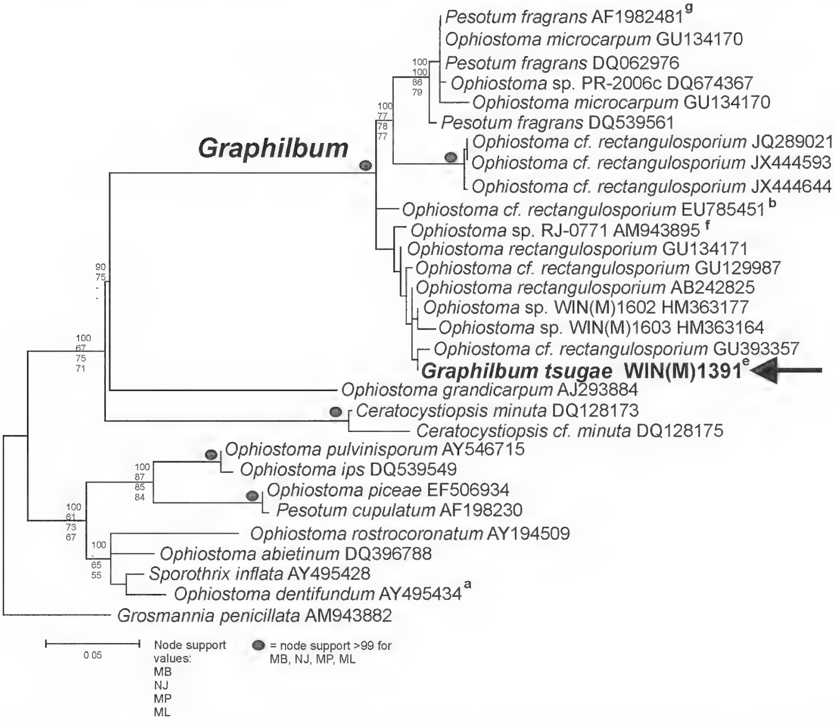

Graphilbum
Pesotum fragrans AF1982481 g
Ophiostoma microcarpum GU134170
Pesotum fragrans DQ062976
Ophiostoma sp. PR-2006c DQ674367
Ophiostoma microcarpum GU134170
Pesotum fragrans DQ539561
Ophiostoma cf. rectangulosporium JQ289021 d
Ophiostoma cf. rectangulosporium JX444593 c
Ophiostoma cf. rectangulosporium JX444644
Ophiostoma cf. rectangulosporium EU785451 b
Ophiostoma sp. RJ-0771 AM943895 f
Ophiostoma rectangulosporium GU134171
Ophiostoma cf. rectangulosporium GU129987
Ophiostoma rectangulosporium AB242825
Ophiostoma sp. WIN(M)1602 HM363177
Ophiostoma sp. WIN(M)1603 HM363164
Ophiostoma cf. rectangulosporium GU393357
Graphilbum tsugae WIN(M)1391 e
Ophiostoma grandicarpum AJ293884
Ceratocystiopsis minuta DQ128173
Ceratocystiopsis cf. minuta DQ128175
Ophiostoma pulvinisporum AY546715
Ophiostoma ips DQ539549
Ophiostoma piceae EF506934
Pesotum cupulatum AF198230
Ophiostoma rostrocoronatum AY194509
Ophiostoma abietinum DQ396788
Sporothrix inflata AY495428
Ophiostoma dentifundum AY495434 a
Grosmannia penicillata AM943882
100 100 86 79
100 77 78 77
90 75 - -
100 67 75 71
100 87 85 84
100 61 73 67
100 - 65 55
0.05
Node support values: MB NJ MP ML
● = node support >99 for MB, NJ, MP, ML

FIG. 3: Phylogenetic tree based on ITS sequence data. Levels of confidence for the major nodes in the phylogenetic tree are based on posterior probability (PP) values (MrBayes; first value), and bootstrap support (BS) in NEIGHBOR (second value), DNAPARS (third value), and Maximum likelihood analysis (fourth value). Nodes that received less than 50% support (BS or PP) were collapsed. Tree topology and branch lengths are based on Bayesian analysis and are proportional to the number of substitutions per site. GenBank accession numbers are listed next to species names.

region in strains identified as *Pesotum fragrans*, *Ophiostoma rectangulisporium*, *Ophiostoma* cf. *rectangulisporium*, and *O. microcarpum* (Fig. 3, Table 1). Comparisons within this ITS data set also strongly suggest that there are several yet to be described cryptic species within this clade. In particular the circumscription of *O. rectangulisporium* and isolates designated as *O.* cf. *rectangulisporium* need to be reevaluated, as the molecular data obtained failed to group these taxa into an appropriate clade relative to their assigned names. All these species are currently expected to be treated as members of the now holomorph genus *Graphilbum* (see below), whose species form a monophyletic group apart from species of *Ophiostoma* sensu stricto.

Taxonomy

Graphilbum tsugae J. Reid & Georg Hausner **sp. nov.** Figs. 4-7

MycoBank MB809811

Differs from all previously described species of *Graphilbum* by its apical ostiolar projections being not true ostiolar hyphae but elongated tapering neck cells with jagged outlines, its rDNA sequences, and its variably solitary or sporodochial anamorphic conidiophores.

Type: Canada, British Columbia, Highway 1, Annis Mountain, near Salmon Arm, from a felled log of *Tsuga heterophylla* (Raf.) Sarg., isolated from spore drop at apex of a perithecium in unidentified bark beetle gallery, 21 September 1987, collected and isolated by J. Reid. (**Holotype**, UAMH 11701 [dried culture on agar plus wood chips], derived from herein designated isotype culture WIN(M) 1391.

Etymology: *tsugae*, referring to the host genus *Tsuga*.

Maximum growth occurring on 2% malt-extract agar amended with yeast extract over a temperature range from 20°C to 25°C; maximum diameter 60 mm after seven days, i.e., relatively slow growing. No measurable growth at 10°C and below or 35°C and above. Colonies remaining only white over this period.

Anamorph apparently pleomorphic on wood chip agar plates. Some conidia developing terminally on short, simple, non-branched to slightly branched conidiophores. Others on variously branched conidiophore clusters that do not develop in any consistent branching pattern. Some are initially hyaline and verticillate, but later secondary cymose branches develop (see Seifert et al. 2011, fig. 100D) arising from beneath a septum in the parent branch. In others, branches that have arisen initially from a common point continue to branch along their length in a cymose fashion. Often these variously branched forms aggregate in clusters on the substrate, and then appear superficially as sporodochia until mounted. Conidiogenous cells proliferating in an imprecisely determined manner, as at times either sympodial or percurrent, or both presumptive forms can be observed in single mounts. Conidia one-celled, hyaline, oblong and parallel-sided in plan view (see Olchowecki &

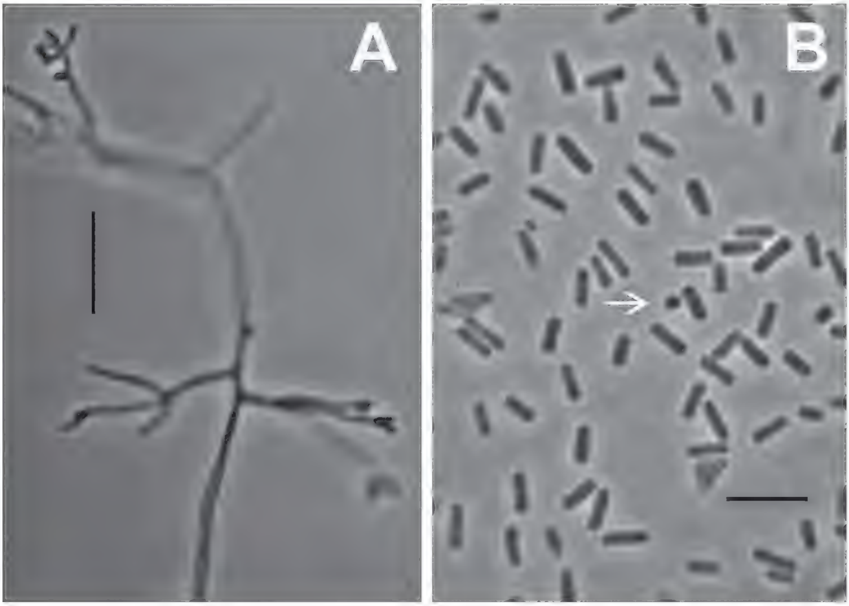


Fig. 4: *Graphilbum tsugae* (wood chip agar cultures). A. Simple conidiophores bearing conidia. B. Conidia in plan view (see Olchowecki & Reid 1974, p.1678 for definition). Scale bars: A = 35 µm; B = 8 µm.

Reid 1974, p. 1678 for definition); generally with slightly rounded ends, but in some cases one end flattened when fully abscised; very uniform in size, cylindrical in cross section (arrow); 3-5(-6) (mean ± s.d. = 4.0 ± 1.05) µm long; frequently produced in slimy masses that occasionally form around the bases of perithecia developing on the wood chips. In such masses the more complicated conidiophores appear.

Mature perithecia black; initials produced abundantly on malt extract supplemented with yeast extract rarely maturing. Abundant mature perithecia developing in two months on plates containing wood chips embedded in the agar. Bases obpyriform, ampulliform, or globose, 75-120 (-168) µm wide (mean ± s.d. = 102 ±16 µm), 75-113 (-140) µm high (mean ± s.d = 94 ± 19 µm). Surface smooth to slightly irregular, often adorned with short dark hair-like hyphae. Neck black, (87-) 138-200 (-280) µm long (mean ± s.d. = 166 ± 49 µm), smooth to occasionally hairy; base 27.5-50 µm wide (mean ± s.d. = 39.1 ± 6.24 µm), tapering to 12.5–25 µm (mean ± s.d. = 18 ± 4.0 µm) just below the point of diverging apical projections; these projections are not ostiolar hyphae, but elongated terminal neck cells, (20-)25-40 µm long (mean ± s.d. = 33.5 ± 7 µm), crooked to jagged cellular extensions that taper to a point, apparently developing by differentiation from normal terminal neck cells; base 2-5 µm

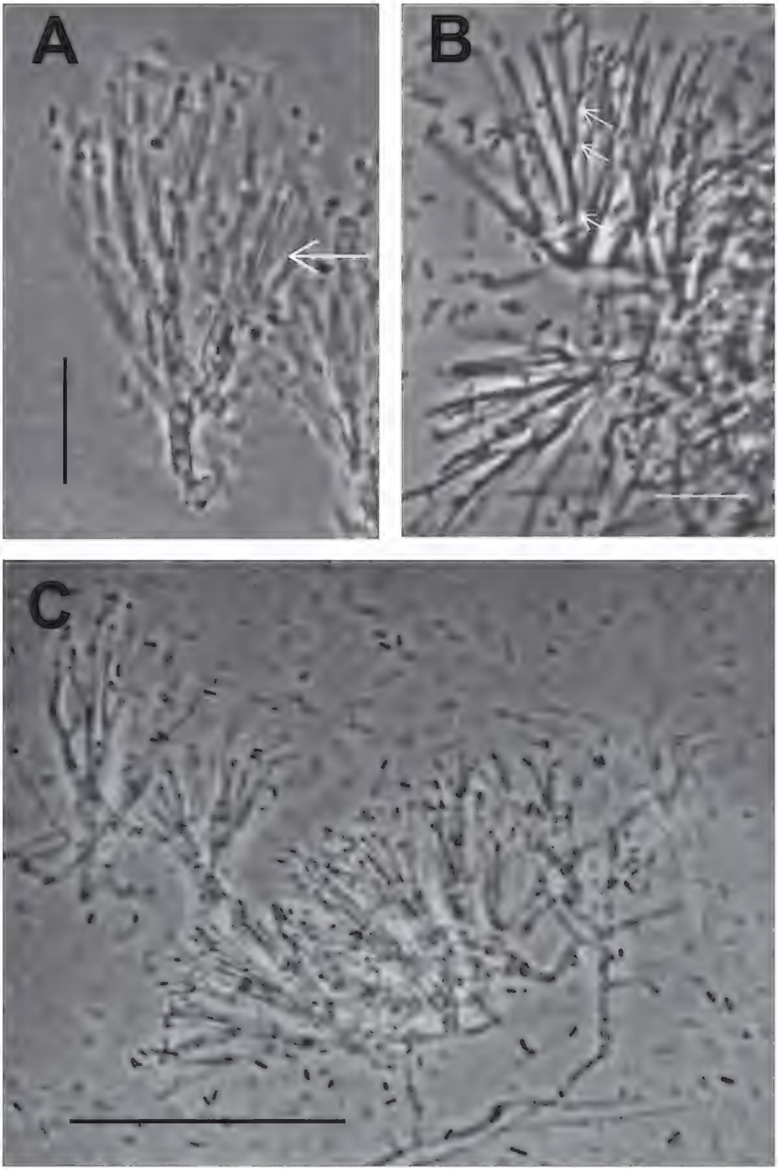
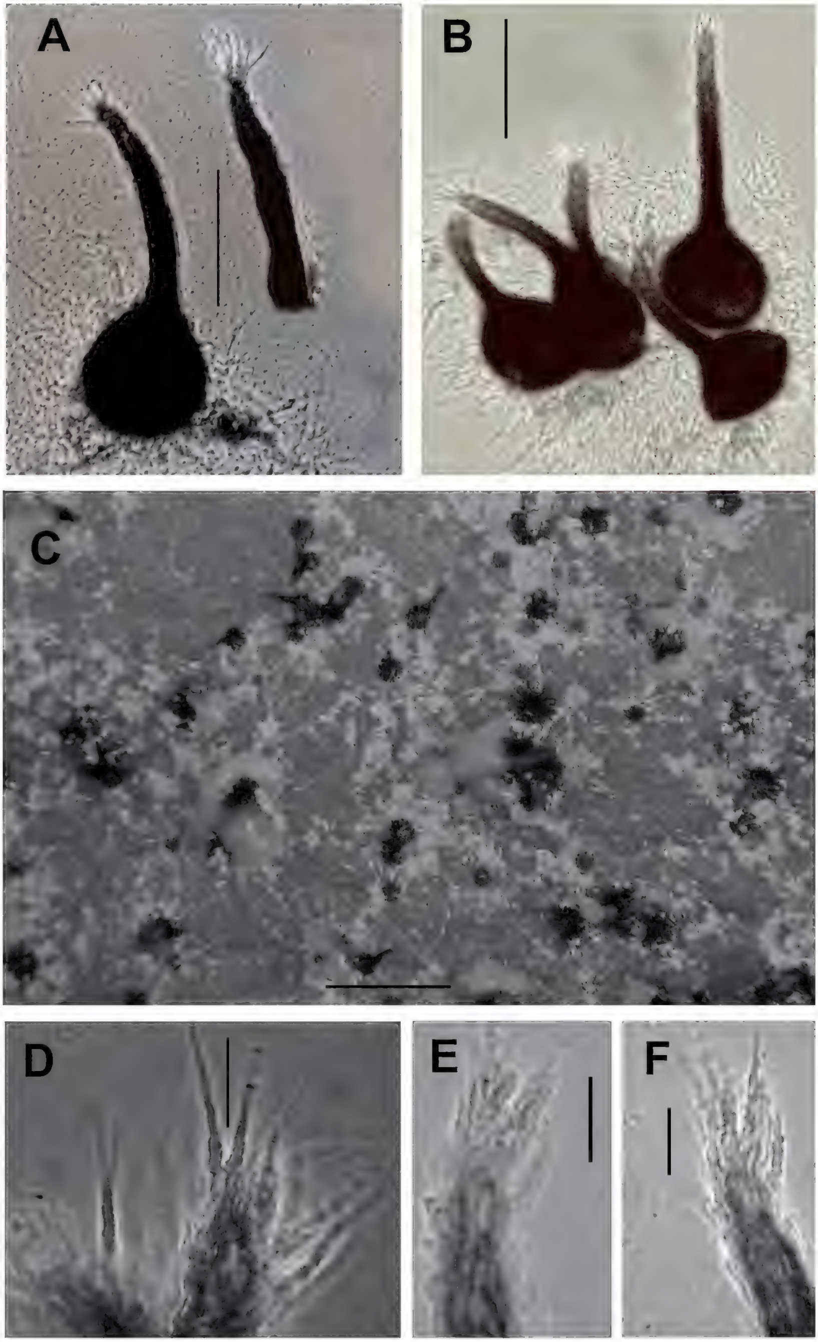


Fig. 5: *Graphilbum tsugae* (wood chip agar cultures). Variously branched conidiophores. A. Single verticillate conidiophore bearing conidia; arising from beneath septum in a parent branch (arrow). B. Portion of a compound aggregation of conidiophores showing successive cymose branches (see Seifert et al. 2011, fig.100D) in a single element (arrows). C. Aggregation of verticillate conidiophores formed in culture on and around the wood chips. Scale bars: A = 12 µm; B = 30 µm; C = 65 µm.


A
B
C
D
E
F

wide (mean ± s.d = 3.5 ± 0.7 µm). Ascospores hyaline, one-celled, oblong to rectangular with slightly rounded ends, rarely slightly constricted in the middle; sheath present but slightly flared and projecting out at the corners in plan view; 3–5 (mean ± s.d. = 3.8 ±. 0.7 × 1.0 µm).

ADDITIONAL MATERIAL EXAMINED: **CANADA, BRITISH COLUMBIA**, Highway 1, Annis Mountain, near Salmon Arm, from a second felled log of *Tsuga heterophylla* at the same location from which the holotype material was obtained, 21 September, 1987. (1) A dried culture grown from isolate WIN(M)1387 (= UAMH 11699] originally isolated aseptically from a slab of stained wood found adjacent to the beetle galleries in the log; (2) A dried culture grown from isolate WIN(M)1384 [= UAMH 11700] that had been isolated from perithecial ooze extruded from a broken perithecium in the beetle galleries in a second small slab of wood taken from this second log. Both samples collected and isolated by J. Reid.

Finally, we correct errors in the citation of:

Graphium rectangulisporium (Ohtaka, Masuya &Yamaoka) Z.W.de Beer & M.J. Wingf. CBS Biodiversity Series 12: 269. 2013 [as "rectangulosporium (R.W. Davidson)"].

≡ *Ophiostoma rectangulisporium* Ohtaka, Masuya & Yamaoka, Can. J. Bot. 84: 290. 2006 [as "*rectangulosporium*"; orthographic correction to comply with McNeill et al. 2012: Art. 60.8].

The miscitation of "R.W. Davidson" as the basionym author appears to have been a transcriptional error.

Discussion

Kim (2010) and Kim et al. (2011) drew attention to a sterile, white, fast growing isolate (GU393357) obtained from *Orthotomicus erosus* (Wollaston) from *Pinus* spp. growing in California that groups closely with our new species (FIG. 3). However, both its reported growth rate and colour range differ markedly from those of *G. tsugae*. We believe both our morphological data and molecular analysis support our isolates as distinct from all other species formerly assigned to *Ophiostoma*. They are, however, linked by molecular criteria to a small group of other former *Ophiostoma* species whose anamorphs had been considered members of the then anamorphic genus *Graphilbum*. This observation is significant due to recent changes proposed by de Beer et al. (2013a, figs. 2 & 3, pp. 4 & 6) and de Beer & Wingfield (2013, figs. 4b & 5b, pp. 29 & 31). In both these papers *Graphilbum* is used as a holomorph-generic name in trees, and in de Beer & Wingfield (2013), the species group to be included in *Graphilbum* is discussed on page 39.

FIG. 6 (left): *Graphilbum tsugae* (wood chip agar cultures). A–C. Mature perithecia. A. Single mature perithecium and a detached perithecial neck. B. Cluster of five mature perithecia. C. Mature perithecia growing on wood chips in agar medium. Note the droplets in which aggregated conidiophores are often found. D– F. Apical neck projections. Scale bars: A, B = 110 µm; C = 650 µm; D = 15 µm; E = 17 µm; F = 10 µm.

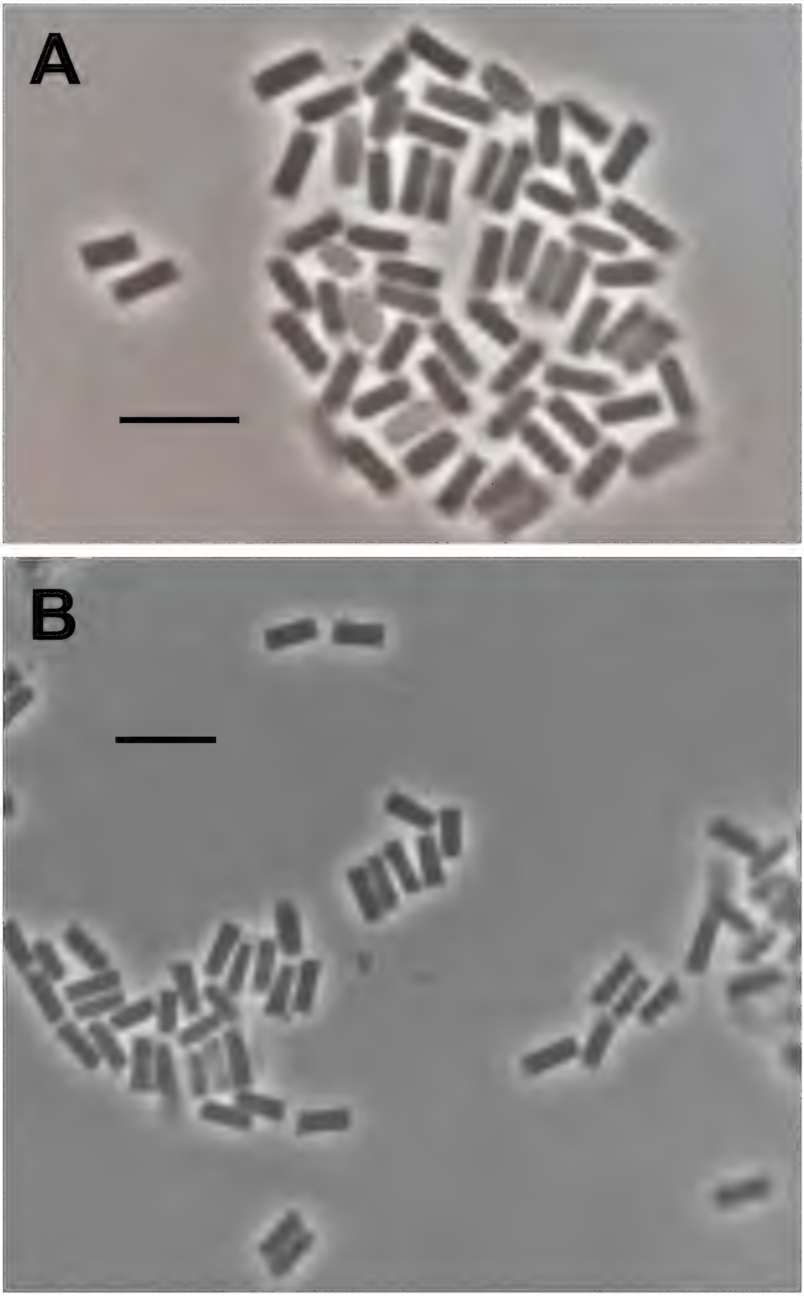


FIG. 7 *Graphilbum tsugae* (wood chip agar cultures). Ascospores. Scale bars: A = 8 µm; B = 7 µm.

De Beer et al. (2013b, p. 268) formally redefined the generic name as:

> *Graphilbum* H.P. Upadhyay & W.B. Kendr., Mycologia 67: 800; 1975 emend. Z.W. de Beer, Seifert & M.J. Wingf. [type species *Gra. sparsum*] = *Ceratocystis* Ellis & Halst. section *Ips* H.P. Upadhyay *pro parte, Monogr. Ceratocystis and Ceratocystiopsis,* p. 70. 1981).

On page 269 discussing *Graphilbum sparsum* H.P. Upadhyay & W.B. Kendr. (1975), de Beer et al. (2013b), also state: … "*Graphilbum sparsum* is the type species of the genus… re-introduced here to accommodate species previously treated in the *P. fragrans* complex … ."

Although this resulted in eight species being formally assigned to the genus *Graphilbum* — for only six of which phylogenetic data exists — there is now a ninth: an anamorphic species placed in *Graphilbum* by phylogenetic data (Romón et al. 2014). However, it is clear that de Beer and Wingfield (2013, p. 39) felt strongly that additional isolates studied by them might well deserve similar placement when further supportive data becomes available. Some such potential species are listed with former *Ophiostoma* spp. discussed in their species review under the name of *Graphilbum* (de Beer & Wingfield 2013, p. 39; figs. 4 b, p. 29, 5b, p. 31) and also seen in de Beer et al. (2013a, fig. 3, p. 6). Using phylogenetic data, three other species represented by new combinations in *Hyalorhinocladiella* proposed by Harrington et al. (2010) — *H. ips, H. macrospora, H. tingens* — were shown to represent anamorphs of three different *Ophiostoma* species (de Beer et al. 2013b).

The foregoing changes were made in conformance with the new rule of fungal nomenclature (McNeill et al. 2012, Article 59, Note 2), "… all legitimate fungal names are treated equally… regardless of the life history stage of the type… ." For a history of the origin of this approach see Hawksworth (2011). Following this practice, we describe our holomorphic isolates as representing a new species of the genus *Graphilbum,* originally an anamorphic name as emended above, even though there are some significant morphological inconsistencies among the species now included therein. Our new species introduction will undoubtedly add to these inconsistencies and to the impetus to erect further new genera.

Our ITS sequence analysis, which showed *Graphilbum tsugae* only distantly related to species accommodated in *Ceratocystiopsis*, *Grosmannia,* and *Ophiostoma,* grouped it in a clade previously referred to as the "*P. fragrans* & *O. rectangulosporium* clade" (Jankowiak 2012). Earlier studies based on ITS region analyses also showed that *P. fragrans* and *O. rectangulisporium* form a distinct lineage among the *Ophiostomatales* (Lu et al. 2009, Paciura et al. 2010, Jankowiak & Kolařík 2010, Jankowiak & Bilański 2013ab, Romón et al. 2014), hinting at possible generic status for these taxa. Based on this history, we feel the ITS data support placing our new species, at least for now, in *Graphilbum.*

Because of our ITS data, we critically compared (Table 2) our new species morphologically only to *O. rectangulisporium*, as described and named in Ohtaka et al. (2006). This comparison clearly separates *G. tsugae* from the only other described *Graphilbum* species to which it could possibly have been assigned.

The morphological character that most sets *G. tsugae* apart from all other described *Graphilbum* species are the projections found at the apex of the perithecial neck; we call these ostiolar projections, for they are certainly unlike the more typical ostiolar hyphae that develop in many other species of *Ophiostoma* sensu stricto.

Ostiolar hyphae are commonly found at the perithecial neck apices of many, but not all, *Ophiostoma* species. However, when present, they are generally easily seen during careful examinations, though in some species they can be quite short. They may also be quite long, straight or curved or even spirally curved, parallel-sided or tapered, convergent or divergent, and septate or aseptate and sometimes may have discrete inflated bases. They often simply appear to be extensions of the parallel hyphae comprising the perithecial neck when the neck is so constructed or modified filamentous extensions of the terminal neck cells that are distinctly cellular in structure.

In *G. tsugae*, perithecial necks are apparently composed of relatively thick-walled, primarily oblong to slightly elongate cells. These may also be irregular in outline, but all abut in an interlocking manner and form serially as necks elongate (Fig. 6E).

As the elongation phase of the neck body slows, these cells begin to separate laterally while continuing to elongate apically and tapering to terminate in a sharp point. To us, they resemble "Zeus's thunderbolt." The individual appendages so formed do not appear to be septate (Fig. 6E). We have never previously seen such ostiolar ornamentations.

Graphilbum, as emended by de Beer et al. (2013b), will now contain ten formally described species and, based on rDNA data, several undescribed ones. *Graphilbum* species either lack conidial states (*O. rectangulisporium*, Ohtaka et al. 2006) or produce anamorphs ranging between hyalorhinocladiella- to pesotum-like in appearance (de Beer and Wingfield 2013).

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